MBHB CASE NO. 98,365-B

METHODS FOR ACCELERATING BONE AND CARTILAGE GROWTH AND REPAIR

Cross Reference

This application is a continuation-in-part of U.S. Provisional Application Serial No. 60/092,653 filed July 13, 1998 and a continuation in part of U.S. Provisional Application Serial No. 60/130,855 filed March 8, 1999.

Field of the Invention

The present invention relates to methods, compositions, and kits for the repair, regeneration, and implantation of bone and cartilage.

Background of the Invention

Natural mechanisms of repair, healing and augmentation are similar for bone and cartilage. (U.S. Patent No. 5,686,116) Although repair, healing and augmentation require a complex series of events that are not well defined, it is known that specific, naturally occurring factors are required to achieve these objectives. Such factors are released or migrate into the injured area, and stimulate osteoblasts and chondrocytes and odontoblasts in bone and cartilage to stimulate matrix formation and remodeling of the wounded area. (ten Dijke et al., Bio/Technology, 7:793-798 (1989))

Living bone tissue is continuously being replenished by the processes of resorption and deposition of bone matrix and minerals. This temporally and spatially coupled process, termed bone remodeling, is accomplished largely by two cell populations, the osteoclasts and

osteoblasts. (U.S. Patent No. 5,656,598, incorporated by reference herein in its entirety) The remodeling process is initiated when osteoclasts are recruited from the bone marrow or the circulation to the bone surface and remove a disk-shaped packet of bone. The bone matrix and mineral is subsequently replaced by a team of osteoblasts recruited to the resorbed bone surface from the bone marrow. Osteoblasts are derived from local mesenchymal (stromal) precursors which differentiate into osteoblasts.

New bone can be formed by three basic mechanisms: osteogenesis, osteoconduction and osteoinduction. (U.S. Patent No. 5,464,439 incorporated by reference herein in its entirety) In osteogenic transplantation, viable osteoblasts and peri-osteoblasts are moved from one body location to another where they establish centers of bone formation. Cancellous bone and marrow grafts provide such viable cells. TGF-beta has been shown to stimulate proliferation and matrix synthesis of osteoblastic cells (Centrella, et al. (1987) J. Biol. Chem. 262:2869-2874) and to inhibit the formation and activity of osteoclastic cells (Chenu, et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:683-5687; Kiebzak et al. (1988) J. Bone Min. Res. 3:439-446), and to stimulate local bone formation in vivo. (Joyce, et al. (1990) J. Cell. Biol. 110:2195-2207; Noda and Camilliere (1989) Endocrinology 124:2991-2294). Other factors reported to stimulate bone growth include bone morphogenetic proteins (WO 88/00205), insulin-like growth factor (IGF) (Endocrinol. Metab. 13:E367-72,1986), and parathyroid hormone (J. Bone & Min. Res. 1:377-381, 1986).

Members of the bone morphogenetic protein family have been shown to be useful for induction of cartilage and bone formation. For example, BMP-2 has been shown to be able to induce the formation of new cartilage and/or bone tissue in vivo in a rat ectopic implant model, see U.S. Pat. No. 5,013,649; in mandibular defects in dogs, see Toriumi et al., Arch. Otolaryngol Head Neck Surg., 117:1101-1112 (1991); and in femoral segmental defects in sheep, see Gerhart

et al., Trans Orthop Res Soc, 16:172 (1991). Other members of the BMP family have also been shown to have osteogenic activity, including BMP-4, -6 and -7 (see Wozney, Bone Morphogenetic Proteins and Their Gene Expression, in Cellular and Molecular Biology of Bone, pp. 131-167 (Academic Press, Inc. 1993)). BMP proteins have also been shown to demonstrate inductive and/or differentiation potentiating activity on a variety of other tissues, including cartilage. (U.S. Patent No. 5,700,774, hereby incorporated by reference in its entirety.

In the transplantation of large segments of cortical bone or allogenic banked bone, direct osteogenesis does not occur. Rather, osteoconduction occurs wherein the dead bone acts as a scaffold for the ingrowth of blood vessels, followed by the resorption of the implant and deposition of new bone. This process is very slow however, often requiring years to reunite a large segmental defect.

Osteoinduction is the phenotypic conversion of connective tissue into bone by an appropriate stimulus. As this concept implies, formation of bone can be induced at even non-skeletal sites. Osteoinduction is preferred over osteoconduction, as grafts of this type are typically incorporated into the host bone within a two-week period. In contrast, osteoconductive grafts have been found to be non-incorporated as long as one year after implantation. In order to provide an environment suitable for osteoinduction, a material should be selected which is not only capable of inducing osteogenesis throughout its volume, but is also biocompatible, non-inflammatory, and possesses the ability to be ultimately resorbed by the body and replaced with new, natural bone.

Among the pathological conditions associated with abnormal bone cell function are osteoporosis, osteoarthritis, Paget's disease, osteohalisteresis, osteomalacia, periodontal disease, bone loss resulting from multiple myeloma and other forms of cancer, bone loss resulting from

side effects of other medical treatment (such as steroids), and age-related loss of bone mass. Inadequate organic matrix mass places an individual at risk of skeletal failure such that bone fractures can result from the minimal trauma of everyday life. Such fractures cause significant illness, or morbidity, inasmuch as there is insufficient repair or healing of the fractures. In certain pathologic conditions, osteoclast-mediated resorption is not regulated by osteoblasts but is driven by cancer cells, infecting organisms or the host's immune cells. In those disease conditions, resorption of bone far exceeds bone formation. Such accelerated osteoclastic activity leads to excessive release of calcium from the inorganic mineral in bone, with a concomitant net loss of skeletal mass, often with an attendant disturbance in calcium homeostasis in the form of elevated blood levels of calcium. (U.S. Patent No. 5,686,116, incorporated by reference herein in its entirety.)

Although methods for directing new bone formation are known, improved methods that provide for accelerated bone growth are needed. For example, currently approved therapeutic agents for osteoporosis are antiresorptives. As such, they are not as effective in patients with established osteoporosis of either type (decreased bone density with fractures of the vertebrae and/or hip), or in patients with Type II osteoporosis. In addition, the most accepted preventive agent for osteoporosis currently in use is estrogen therapy, which is not an acceptable therapeutic agent for women with a history of breast cancer or endometrial cancer or for men with osteoporosis.

Similarly, successful implantation and function of bone implants depends on bonding of the adjacent bone to the implant. (U.S. Patent No. 5,686,116) Such bonding requires bone repair by the formation of new matrix components at the interface between the implant and the bone proximate to the implant. An estimated ten percent of bone and joint prosthetic devices that are

placed in people fail to function due to non-bonding of the bone to an implant. The resulting disability often requires reoperation and reimplantation of the device. Furthermore, five to ten percent of all bone fractures are never repaired. Although many methods have been proposed to cure these non-healing bone fractures, none has yet proven to be satisfactory. Based on all of the above, there clearly exists a need in the art for improved methods that provide for accelerated bone growth.

Cartilage is a specialized type of dense connective tissue consisting of cells embedded in a matrix. There are several kinds of cartilage. (U.S. Patent No. 5,736,372, herein incorpoirated by reference in its entirety.) Translucent cartilage having a homogeneous matrix containing collagenous fibers is found in articular cartilage, in costal cartilages, in the septum of the nose, in larynx and trachea. Articular cartilage is hyaline cartilage covering the articular surfaces of bones. Costal cartilage connects the true ribs and the sternum. Fibrous cartilage contains collagen fibers. Yellow cartilage is a network of elastic fibers holding cartilage cells which is primarily found in the epiglottis, the external ear, and the auditory tube. Cartilage is tissue made up of extracellular matrix primarily comprised of the organic compounds collagen, hyaluronic acid (a proteoglycan), and chondrocyte cells, which are responsible for cartilage production. Collagen, hyaluronic acid and water entrapped within these organic matrix elements yield the unique elastic properties and strength of cartilage. Chondrocytes produce both Type I and Type II collagens. Type II collagen is not found in bone, whereas Type I collagen is found in bone. (U.S. Patent No. 5,686,116) It has previously been shown that the endogenous growth factors TGF beta and BMP induce both new cartilage and bone formation. Wozney et al. Science, 242:1528-1533 (1988) and Sporn et al. J. Cell Biol. 105:1039-1045 (1987).

In cartilage, collagen synthesis is required for repair, healing and augmentation, as well as for the successful bonding of grafts and prosthetic devices. (U.S. Patent No. 5,686,116) Collagen is the major structural protein responsible for the architectural integrity of cartilage. Thus, an adequate supply of chondrocytes is essential in order to produce sufficient amounts of collagen for repair, healing, and augmentation of cartilage. Other, noncollagen proteins, such as osteonectin, fibronectin and proteoglycans are also important for cartilage repair.

Cells such as synoviocytes that are found in joint spaces adjacent to cartilage have an important role in cartilage metabolism. Synoviocytes produce metallo-proteinases, such as collagenases that are capable of breaking-down cartilage. TGF beta is known to inhibit cell-release (and probably synthesis) of metallo-proteinases and to induce chondrocytes (cartilage forming cells) to produce new matrix components and inhibit production of cartilage destructive enzymes so as to effect cartilage repair, healing and augmentation. Sporn et al. (1987). It has also been shown that mice deficient in parathyroid hormone-related peptide (PTHrP) exhibit abnormal cartilage maturation, indicating that PTHrP is an essential factor for chondrocyte development and maturation. (U.S. Patent No. 5,700,774)

Cartilage implants are often used in reconstructive or plastic surgery such as rhinoplasty. There is a need in the art for methods that increase chondrocyte proliferation and collagen synthesis, and thus inhibit cartilage destruction and enhance cartilage repair. Such methods would increase the clinical utility of cartilage repair including but not limited to cartilage grafts and healing of cartilage grafts.

Although some of the above methods have met with limited success, there remains a need in the art for improved methods for enhancing bone and cartilage repair, healing and augmentation, and for enhancing the attachment and fixation of bone and cartilage implants.

Summary of the Invention

The present invention provides methods, kits, and compositions for 1) enhancing bone and cartilage repair; 2) bone and prosthesis implantation; 3) attachment and fixation of cartilage to bone or other tissues; and methods, cell culture medium and kits for the proliferation of chondrocytes; all of which comprise the administration of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, angiotensin II (AII), AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists.

These aspects and other aspects of the invention become apparent in light of the following detailed description.

Brief Description of the Figures

Figure 1 is a bar graph showing the effect of AII, AIII, and GSD37B (10 μg/ml) on chondrocyte proliferation.

Figure 2 is a bar graph showing the effect of AII, GSD36, GSD37B, GSD38B, and GSD28 (10 μg/ml) of the invention on chondrocyte proliferation.

Figure 3 is a bar graph showing the effect of AII, 1GD, 2GD, and 3GD (10μg/ml) on chondrocyte proliferation.

Figure 4 is a bar graph showing the effect of AII, AII(1-7), GSD22A, GSD24B, and GSD28 (10 μg/ml) of the invention on chondrocyte proliferation.

Detailed Description of the Preferred Embodiments

All references, patents and patent applications are hereby incorporated by reference in their entirety.

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

As defined herein the phrase "enhancing bone repair" refers to increasing the rate of new bone formation via bone remodeling, osteogenesis, osteoconduction and/or osteoinduction. The methods for enhancing bone repair in a mammal of the invention include those that stimulate bone formation and those that reverse bone loss. The methods can thus be used for (1) providing a subject with an amount of a substance sufficient to act prophylactically to prevent the development of a weakened and/or unhealthy state; or (2) providing a subject with a sufficient amount of a substance so as to alleviate or eliminate a disease state and/or the symptoms of a disease state, and a weakened and/or unhealthy state.

As used herein the term "enhancing cartilage repair" comprises healing and regeneration of cartilage injuries, tears, deformities or defects, and prophylactic use in preventing damage to cartilaginous tissue.

The present invention fulfills the need for methods to enhance bone repair in a mammal suffering from bone fractures, defects, and disorders which result in weakened bones such as

osteoporosis, osteoarthritis, Paget's disease, osteohalisteresis, osteomalacia, periodontal disease, bone loss resulting from multiple myeloma and other forms of cancer, bone loss resulting from side effects of other medical treatment (such as steroids), and age-related loss of bone mass. In addition, bony ingrowth into various prosthetic devices can be greatly enhanced so that such artificial parts are firmly and permanently anchored into the surrounding skeletal tissue through a natural osseous bridge.

The present invention further fulfills the need for methods to enhance the repair of cartilage in a mammal, by accelerating the proliferation of chondrocytes and thereby increasing the synthesis of collagen for use in cartilage repair. Such methods have application in the healing of cartilage, for example articular cartilage tears, deformities and other cartilage defects in humans and other animals. The methods have prophylactic use in preventing damage to cartilaginous tissue, as well as use in the improved fixation of cartilage to bone or other tissues, and in repairing defects to cartilage tissue. De novo cartilaginous tissue formation induced by the compounds of the present invention contributes to the repair of congenital, trauma induced, or other cartilage defects of other origin, and is also useful in surgery for attachment or repair of cartilage. The methods and compositions of the invention may also be useful in the treatment of arthritis and other cartilage defects. The methods of the present invention can also be used in other indications wherein it is desirable to heal or regenerate cartilage tissue. Such indications include, without limitation, regeneration or repair of injuries to the articular cartilage. The methods of the present invention provide an environment to attract cartilage-forming cells, stimulate growth of cartilage-forming cells or induce differentiation of progenitors of cartilage-forming cells and chondrocytes.

The methods and kits of the present invention also provide improved chemically defined medium for accelerating the proliferation of chondrocytes (cartilage-forming cells). In another embodiment, the compositions and methods of the present invention can be used to treat chondrocytic cell lines, such as articular chondrocytes, in order to maintain chondrocytic phenotype and survival of the cells. The treated cell populations are therefore also useful for gene therapy applications.

U.S. Patent No. 5,015,629 to DiZerega (the entire disclosure of which is hereby incorporated by reference) describes a method for increasing the rate of healing of wound tissue, comprising the application to such tissue of angiotensin II (AII) in an amount which is sufficient for said increase. The application of AII to wound tissue significantly increases the rate of wound healing, leading to a more rapid re-epithelialization and tissue repair. The term AII refers to an octapeptide present in humans and other species having the sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe [SEQ ID NO:1]. The biological formation of angiotensin is initiated by the action of renin on the plasma substrate angiotensinogen (Clouston et al., *Genomics* 2:240-248 (1988); Kageyama et al, *Biochemistry* 23:3603-3609; Ohkubo et al., *Proc. Natl. Acad. Sci.* 80:2196-2200 (1983); each reference hereby incorporated in its entirety). The substance so formed is a decapeptide called angiotensin I (AI) which is converted to AII by the angiotensin converting enzyme (ACE) which removes the C-terminal His-Leu residues from AI [SEQ ID NO: 37]. AII is a known pressor agent and is commercially available.

Studies have shown that AII increases mitogenesis and chemotaxis in cultured cells that are involved in wound repair, and also increases their release of growth factors and extracellular matrices (diZerega, U.S. Patent No. 5,015,629; Dzau et. al., *J. Mol. Cell. Cardiol.* 21:S7 (Supp III) 1989; Berk et. al., *Hypertension* 13:305-14 (1989); Kawahara, et al., *BBRC* 150:52-9 (1988);

Naftilan, et al., *J. Clin. Invest.* 83:1419-23 (1989); Taubman et al., *J. Biol. Chem.* 264:526-530 (1989); Nakahara, et al., *BBRC* 184:811-8 (1992); Stouffer and Owens, *Circ. Res.* 70:820 (1992); Wolf, et al., *Am. J. Pathol.* 140:95-107 (1992); Bell and Madri, *Am. J. Pathol.* 137:7-12 (1990). In addition, AII was shown to be angiogenic in rabbit corneal eye and chick chorioallantoic membrane models (Fernandez, et al., *J. Lab. Clin. Med.* 105:141 (1985); LeNoble, et al., Eur. J. Pharmacol. 195:305-6 (1991). Additionally, AII and angiotensin III analogs and fragments thereof have been shown to be effective in wound healing. (U.S. Patent No. 5,629,292; International Application No. WO 95/08565; International Application WO 95/08337; International Application No. WO 96/39164; all references hereby incorporated in their entirety.)

Previous studies have suggested that angiotensin I (AI) and AII both stimulate bone resorption *in vitro* by osteoclasts incubated on bone slices, but only in the presence of osteoblastic cells, suggesting that the effect of angiotensin II was not direct, but rather is mediated by a primary hormonal interaction on cells of the osteoblastic lineage. (Hatton et al., *J. Endocrinol.* 152:5-10 (1997)). AI stimulation of bone resorption was inhibited by ACE inhibitors, suggesting that the formation of AII from AI was responsible for the stimulation of bone resorption. Neither AI nor AII were shown to have any effect on osteoclast formation. Thus, this study suggests that local bone destruction may be mediated by AII's stimulation of bone resorption.

Other studies have demonstrated AII stimulation of DNA and collagen synthesis *in vitro* on primary cultures of isolated, phenotypically immature osteoblasts derived from the periosteum of fetal rat calvaraiae and human adult trabecular bone. (Lamparter et al., *J. Cell. Physiol.* 175:89-98 (1998)) No direct AII effect was detected on primary cell populations with a mature osteoblast phenotype, and an indirect effect through AII-responsive osteoblastic

precursor cells was proposed. Similar *in vitro* studies on osteoblast-rich populations of cells demonstrated a similar effect, while not ruling out stimulation of mature osteoblast proliferation. (Hiruma et al., *Biochem and Biophys. Res. Commun.* 230:176-178 (1997)) Another study suggests that AII may decelerate the differentiation and bone formation of rat calvarial osteoblasts. (Hagiwara et al., J. of Endocrinology 156:543-550 (1998))

Based on all of the above studies, there is no expectation that the use of angiotensinogen, angiotensin I (AI), AI analogues, AI fragments and analogues thereof, AII, AII analogues, AII fragments or analogues thereof or AII AT_2 type 2 receptor agonists would be effective in enhancing bone and cartilage repair, or effective in accelerating chondrocyte proliferation and collagen synthesis.

Previous studies in our laboratory have demonstrated that a class of AII and AII analogues and fragments stimulate the proliferation of mesenchymal stem cells, which give rise to the cells that make up bone and cartilage. (U.S Patent Application 09/012,400, filed January 23, 1998, herein incorporated by reference in its entirety.)

A peptide agonist selective for the AT2 receptor (AII has 100 times higher affinity for AT2 than AT1) has been identified. This peptide is p-aminophenylalanine 6-AII ["(p-NH_2-Phe)6-AII)"], Asp-Arg-Val-Tyr-Ile-Xaa-Pro-Phe [SEQ ID NO.36] wherein Xaa is p-NH_2-Phe (Speth and Kim, BBRC 169:997-1006 (1990). This peptide gave binding characteristics comparable to AT2 antagonists in the experimental models tested (Catalioto, et al., *Eur. J. Pharmacol.* 256:93-97 (1994); Bryson, et al., *Eur. J. Pharmacol.* 225:119-127 (1992).

The effects of AII receptor and AII receptor antagonists have been examined in two experimental models of vascular injury and repair which suggest that both AII receptor subtypes (AT1 and AT2) play a role in wound healing (Janiak et al., *Hypertension* 20:737-45 (1992);